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Review Article

Formation of Toxic Amyloid Fibrils by Amyloid β-Protein on Ganglioside Clusters

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It is widely accepted that the conversion of the soluble, nontoxic amyloid β-protein (Aβ) monomer to aggregated toxic Aβ rich in β-sheet structures is central to the development of Alzheimer's disease. However, the mechanism of the abnormal aggregation of Aβ in vivo is not well understood. Accumulating evidence suggests that lipid rafts (microdomains) in membranes mainly composed of sphingolipids (gangliosides and sphingomyelin) and cholesterol play a pivotal role in this process. This paper summarizes the molecular mechanisms by which Aβ aggregates on membranes containing ganglioside clusters, forming amyloid fibrils. Notably, the toxicity and physicochemical properties of the fibrils are different from those of Aβ amyloids formed in solution. Furthermore, differences between Aβ-(1–40) and Aβ-(1–42) in membrane interaction and amyloidogenesis are also emphasized.

1. Introduction

It is widely accepted that the amyloid β-protein (Aβ), which exists in fibrillar forms as a major component of senile plaques, is central to the development of Alzheimer's disease (AD) [1, 2]. The conversion of soluble, nontoxic Aβ monomer to aggregated toxic Aβ rich in β-sheet structures ignites the neurotoxic cascade(s) of Aβ [3]. The mechanism of the abnormal aggregation of Aβ is not well understood. The physiological concentration of Aβ in biological fluids ($<10^{-8}$ M) [4] is much lower than the concentration ($\sim 1\,\mu$M) above which Aβ-(1–40) spontaneously forms fibrils [5]. Therefore, there should be mechanisms that facilitate the abnormal aggregation of Aβ under pathological conditions. Although clusterin (Apo J) [6] and Zn^{2+} ions [7] were reported to facilitate the aggregation of Aβ more than a decade ago, their aggregation-promoting mechanisms are yet to be elucidated. In addition to these soluble factors, Jarrett and Lansbury, Jr. suggested that Aβ fibrillizes via a nucleation-dependent polymerization mechanism and lipids could act as heterogeneous seeds for the polymerization [8]. In 1995, Yanagisawa and colleagues discovered a specific form of Aβ bound to monosialoganglioside GM1 (GM1) in brains exhibiting early pathological changes of AD and suggested that the GM1-bound form of Aβ may serve as a seed for the formation of toxic amyloid aggregates/fibrils [9].

We have been investigating the interaction of Aβ with ganglioside-containing membranes for a dozen years and found that not the uniformly distributed but the clustered gangliosides mediate the formation of amyloid fibrils by Aβ, the toxicity and physicochemical properties of which are different from those of Aβ amyloids formed in solution. This review article summarizes Aβ-ganglioside interaction in detail, including latest findings that were not covered in our previous reviews [10, 11]. Especially, differences between Aβ-(1–40) and Aβ-(1–42) in membrane interaction and amyloidogenesis are extensively discussed. Furthermore, a link between Aβ aggregation and lipid metabolism is emphasized. It will shed light on one of the initiation processes of AD.

2. Specific Binding of Aβ to Ganglioside Clusters

Early studies indicated that Aβ-(1–40) associates with GM1 in egg yolk phosphatidylcholine (PC) vesicles only when the GM1 content exceeds 30% [12, 13]. The threshold GM1 content is lowered in a sphingomyelin (SM)/cholesterol mixture [13]. These findings suggest that GM1 molecules only in a specific state can recognize Aβ. To reveal the underlying mechanism, we systematically investigated the interaction of dye-labeled-Aβ-(1–40) [14–16] and -Aβ-(1–42) [17] with membranes of various lipid compositions. The N-termini

Table 1: Parameters for the binding of DAC-Aβs to GM1/cholesterol/SM (1 : 1 : 1) LUVs at 37°C.

Aβ	K (10^6 M^{-1})[a]	x_{max} [b](Aβ/GM1, mol/mol)	Ref.
DAC-Aβ-(1–42)	11.1 ± 2.4	0.0361 ± 0.0021	[17]
DAC-Aβ-(1–40)	8.6 ± 3.6	0.0313 ± 0.0041	[16]
DAC-Aβ-(1–28)	0.0184 ± 0.0007	0.0313[c]	[16]

[a] Binding constant.
[b] Maximal value of x (bound Aβ per exofacial GM1, mol/mol).
[c] Assumed to be the same as that of DAC-Aβ-(1–40).

of Aβs were labeled with the 7-diethylaminocoumarin-3-carbonyl group (DAC-Aβ). DAC-Aβ is useful for fluorometrically monitoring protein-lipid interactions, because a significant blue shift and an enhancement in intensity are induced by a change in polarity upon membrane binding. DAC-Aβs do not bind to major membrane lipids, including electrically neutral PC, SM, cholesterol, negatively charged phosphatidylserine, and phosphatidylglycerol under physiological conditions. On the other hand, the proteins exhibit similar high affinity (binding constant ~10^7 M^{-1}) for raft-like membranes composed of GM1, cholesterol, and SM [14, 15]. DAC-Aβ-(1–28) also has a weak affinity for the membrane [16]. Binding parameters are summarized in Table 1. DAC-Aβ-(1–40) also binds to other gangliosides (GD1a, GD1b, GT1b, and asialo GM1) and lactosyl ceramide in raft-like membranes with higher affinity for lipids having larger sugar chains [15, 16]. We have proposed that Aβs specifically bind to ganglioside clusters because a GM1 cluster is formed in GM1/SM/cholesterol membranes but not in GM1/PC membranes. The clustering is facilitated by cholesterol [14].

3. Fibrillization by Aβ on Ganglioside Clusters

Aβ-(1–40) bound to ganglioside clusters assumes different conformations depending on the protein density on the membrane. Circular dichroism measurements revealed that the protein forms an α-helix-rich structure at lower protein-to-ganglioside ratios (≤0.025) whereas it changes its conformation to a β-sheet-rich structure at higher ratios (≥0.05) [14, 15]. Aβ-(1–42) also undergoes similar conformational changes [17]. Only the β-sheet form facilitates amyloidogenesis by Aβ-(1–40) [15, 18–20].

Despite very similar initial protein-ganglioside interaction, that is, the binding behavior and the α-helix-to-β-sheet conformational change, a large difference was observed in amyloidogenic activity (amount of amyloids formed under certain conditions) between Aβ-(1–40) and Aβ-(1–42) [17]. Aβs were incubated with GM1/cholesterol/SM liposomes at a Aβ-to-GM1 ratio of 5, and the aggregation of Aβ was monitored as an increase in fluorescence of the amyloid-specific dye thioflavin-T (Th-T) (Figure 1). Aβ-(1–42) formed amyloids without a lag time at 5 μM. In contrast, Aβ-(1–40) at 5 μM did not form amyloids, at least not in 12 h. At a 10-fold higher concentration, Aβ-(1–40) started to aggregate after a lag time of ~2 h. The effectiveness of Aβ-(1–42) in fibrillogenesis is at least partly due to the fragility

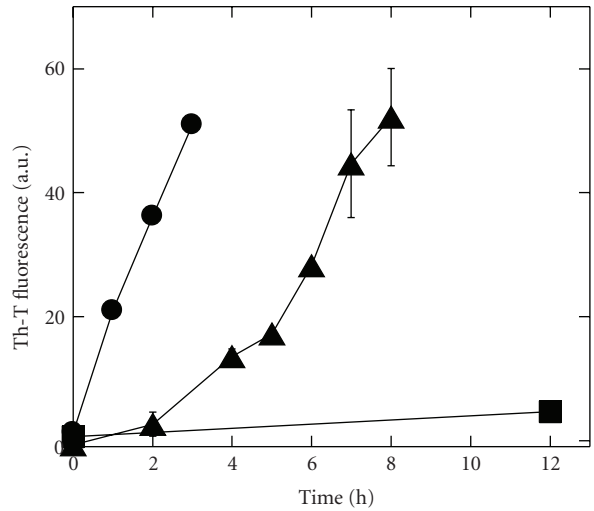

Figure 1: Aβ aggregation in the presence of raft-like liposomes. Aβs (5 μM or 50 μM) were incubated with GM1/cholesterol/SM (1 : 1 : 1) small unilamellar vesicles at a GM1-to-Aβ ratio of 5 at 37°C without agitation, and the aggregation was monitored by the Th-T assay. Symbols: circles, 5 μM Aβ-(1–42); squares, 5 μM Aβ-(1–40); triangles, 50 μM Aβ-(1–40). Data taken from [17].

of fibrils, because the fragmentation greatly facilitates fibril growth [21] (see also Section 5). Other factors, such as the rapid formation of seeds and/or elongation, may also contribute to the difference.

Cell experiments also support the above mechanism of Aβ-ganglioside interaction [22, 23]. Aβ-(1–42) was incubated with neuronal rat pheochromocytoma PC12 cells. Amyloids and gangliosides were detected by the amyloid-specific dye Congo red and the fluorescent-labeled cholera toxin B subunit, respectively. Amyloids were selectively formed on ganglioside-rich domains (Figure 2(a)). Depletion of cholesterol, either by methyl-β-cyclodextrin or the cholesterol synthesis inhibitor compactin, suppressed the accumulation of Aβ. The amyloidogenic activity of Aβ-(1–42) was again more than 10-fold that of Aβ-(1–40) on human SH-SY5Y neuroblastoma cells expressing gangliosides (Figure 2(b)). When cells were incubated with 5 μM Aβ-(1–42), Congo red-positive spots appeared later at 24 h and became prominent with time. In contrast, when cells were incubated with 5 μM Aβ-(1–40), no fibrils were detected even after 72 h. Incubation with a 10-fold higher concentration of the protein, however, resulted in the appearance of Congo red-positive spots at 48 h.

4. Properties of Aβ Fibrils Formed on Ganglioside Clusters

The Aβ fibrils formed on ganglioside clusters (Mem-fibrils) are not identical to those formed in solution (Sol-fibrils) in terms of physicochemical properties and cytotoxicity [20]. Transmission electron micrographs indicate that Mem-fibrils are typical nonbranched fibrils (12.0 ± 0.7 nm, width) whereas Sol-fibrils are thinner fibrils or protofilaments

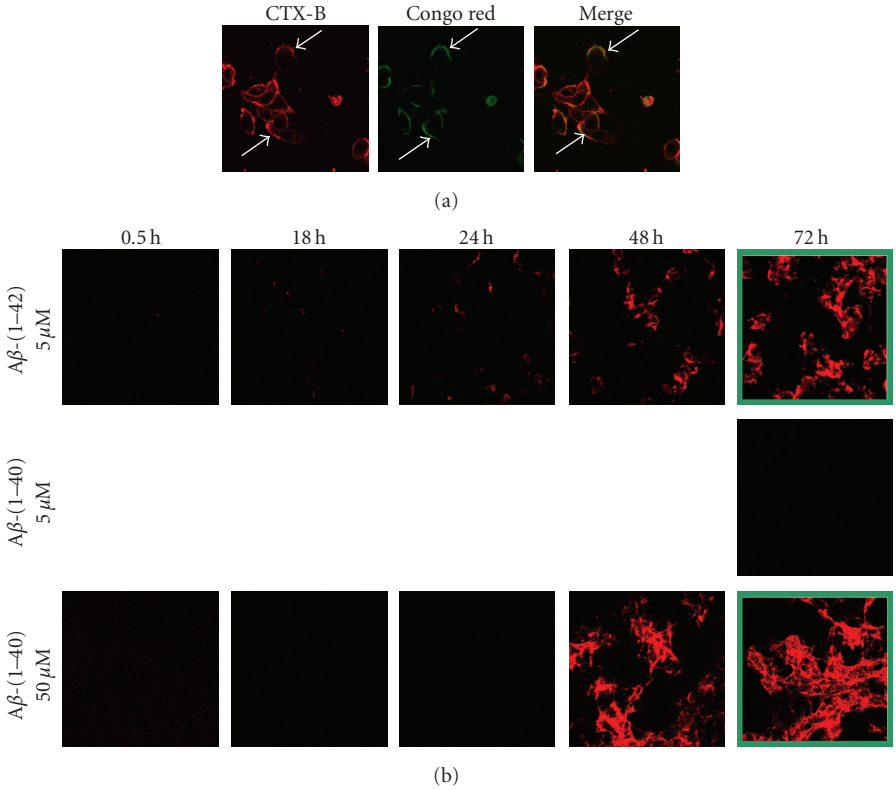

Figure 2: Aβ aggregation on living neuronal cells. (a) Aβ-(1–42) (10 μM) was incubated with PC12 cells for 24 h at 37°C. The distribution of GM1 was detected by using the cholera toxin B subunit conjugated with Alexa Fluor 647 dye (CTX-B, left). Amyloids were visualized by the amyloid-specific dye Congo red (middle). The merging of the two images shows that amyloids were formed in the vicinity of GM1-rich domains of cell membranes (right). Data taken from [10]. (B) Ganglioside-expressing SH-SY5Y cells were incubated with 5 μM Aβ-(1–42) (top), 5 μM Aβ-(1–40) (middle), or 50 μM Aβ-(1–40) (bottom) for 0.5, 18, 24, 48, or 72 h, and the formation of amyloids was detected with Congo red. The conditions under which cell death was observed are framed in green. Data taken from [17].

(6.7 ± 1.3 nm, width), and the protofilaments associate laterally and twist into rope-like fibrils (14.5 ± 0.9 nm, width). The surface hydrophobicity of Mem-fibrils as estimated by the binding of 1-anilinonaphthalene-8-sulfonate (ANS) is larger than that of Sol-fibrils (Figure 3(a)), therefore Mem-fibrils exhibit significantly stronger binding to cell membranes than Sol-fibrils (Figure 3(b)). Consequently, Mem-fibrils are cytotoxic whereas Sol-fibrils are much less toxic (Figure 3(c)). Recently, a correlation between ANS-binding and cytotoxicity was reported for various amyloid species [24].

The structure of Mem-fibrils is suggested to be different from that of Sol-fibrils, in which the cross-β unit is a double-layered structure, with in-resister parallel β-sheets formed by residues 12–24 and 30–40 [25]. The amide I spectrum of the former shows, in addition to a major peak around 1630 cm^{-1} characteristic of a β-sheet, a weak peak at 1695 cm^{-1} whereas that of the latter shows a peak around 1660 cm^{-1} [26].

5. Mechanism of Cytotoxicity by Aβ Fibrils Formed on Ganglioside Clusters

The mechanisms of Aβ-induced cytotoxicity have been controversial. Aβ fibrils were reported to trigger functional disorder in neuronal cells and cell death [27–31] whereas soluble Aβ oligomers have been proposed to play a pivotal role in the onset of AD [6, 28, 32–39]. To obtain an insight into the cytotoxic mechanism of Aβ, we established a multistaining visualization method using unlabeled Aβs and antibodies [17] in contrast to conventional methods using fluorophore-labeled proteins [23, 40]. The accumulation of Aβ, the formation of amyloid fibrils, the formation of oligomers, and cell viability were visualized using the Aβ monoclonal antibody 6E10, the amyloid-staining dye Congo red [22], the antioligomer antibody A11 [34], and calcein acetoxymethyl, respectively. Cell death was detected after the significant accumulation of fibrils (Figure 2(b)) and no A11-positive spot was detected, suggesting that fibril-induced physicochemical stress, such as the induction of a negative curvature [13] or membrane deformation upon fibril growth [41], leads to cytotoxicity. A11-positive oligomers were not formed in the fibrillization with GM1-containing liposomes either [20]. It should be noted, however, that at certain GM1 contents GM1-liposomes generate toxic soluble Aβ-(1–40) oligomers [42]. For both Aβs, similar levels of fibrils were required for cytotoxicity (Figure 2(b)), indicating that the fibrils possess comparable intrinsic toxicity. The fibrillization process and cytotoxicity can be effectively

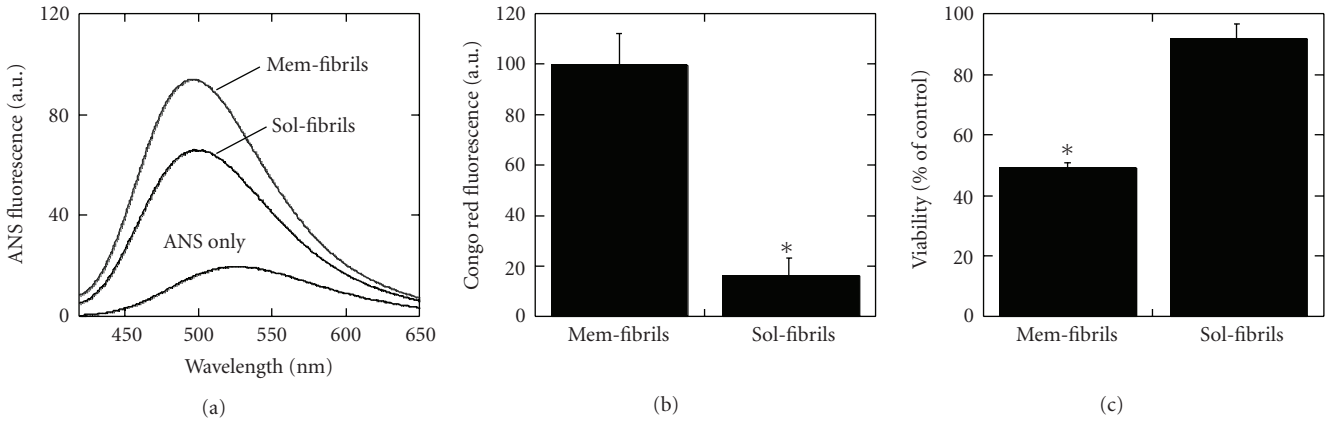

FIGURE 3: Comparison between Mem-fibrils and Sol-fibrils. Data taken from [20]. (a) Fluorescence spectra of ANS (5.0 μM) in PBS were measured in the absence or presence of Mem-fibrils and Sol-fibrils of Aβ-(1–40) (2.5 μM) with an excitation wavelength of 350 nm. The binding of the dye to a hydrophobic surface results in an enhancement in fluorescence intensity. (b) Aβ-(1–40) fibrils (25 μM) were incubated with NGF-differentiated PC12 cells for 30 min. Binding of Aβ-(1–40) fibrils to cells was evaluated by fluorescence intensity of Congo red per cell (mean ± S.E.; $n \sim 100$, $^*P < .001$). (c) Aβ-(1–40) fibrils (25 μM) were incubated with NGF-differentiated PC12 cells for 24 h. Aβ cytotoxicity was estimated with fluorescence intensity of the live cell marker calcein (mean ± S.E.; $n = 6$; $^*P < .001$ against vehicle treatment).

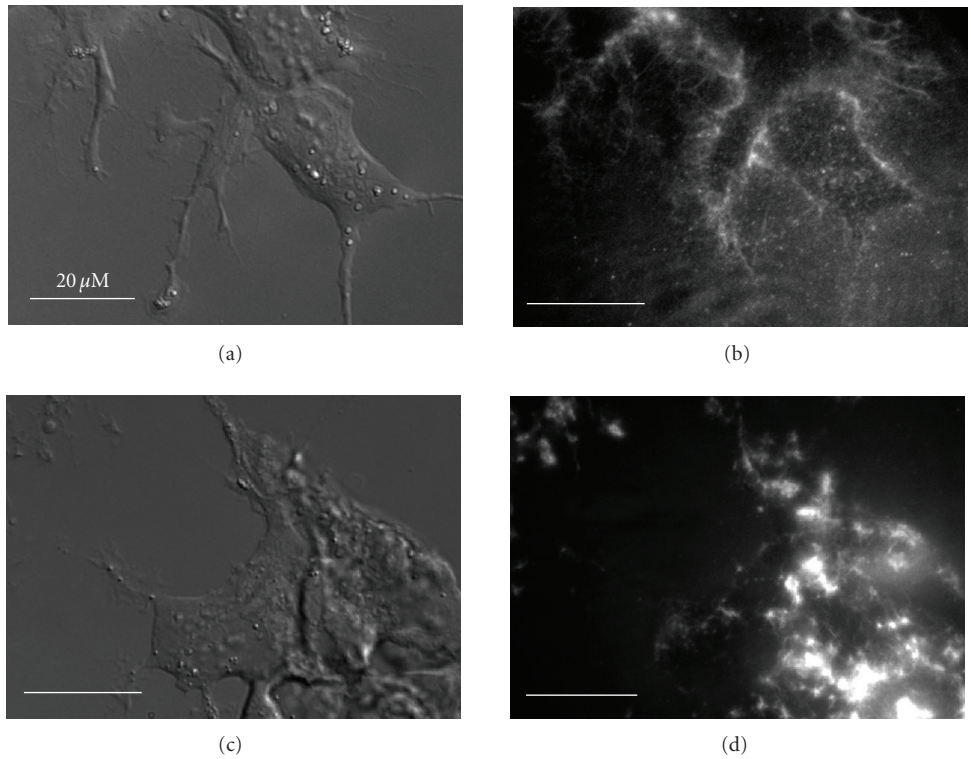

FIGURE 4: Visualization of amyloid fibrils formed on cell membranes using TIRFM. SH-SY5Y cells were treated with 50 μM Aβ-(1–40) ((a), (b)) or 5 μM Aβ-(1–42) ((c), (d)) for 48 h. Amyloid fibrils were stained with 20 μM Congo red. (a) and (c) are DIC images, while (b) and (d) are TIRF images. Data taken from [17].

blocked by small compounds, such as nordihydroguaiaretic acid and rifampicin [19].

The morphology of amyloid fibrils formed on cell membranes was visualized by total internal reflection fluorescence microscopy (TIRFM) [17]. TIRFM effectively reduces the background fluorescence and therefore is suitable for observing the cell surface. Fibrils were stained with Congo red. Relatively long fibrillar structures were detected around the cell membrane for Aβ-(1–40) whereas relatively short fibrils were coassembled in the case of Aβ-(1–42) (Figure 4). The

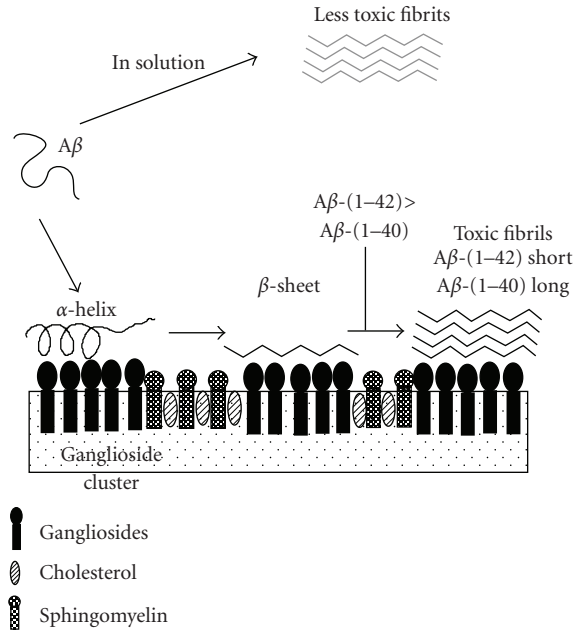

Figure 5: A model for the formation of toxic amyloid fibrils by amyloid β-protein on ganglioside clusters. Aβ is essentially soluble, and takes an unordered structure in solution. Once ganglioside clusters are generated, Aβ binds to the clusters, forming an α-helix-rich structure at lower protein-to-ganglioside ratios whereas the protein changes its conformation to a β-sheet at higher ratios. The β-sheet form facilitates the fibrillization of Aβ, leading to cytotoxicity. The amyloidogenic activity of Aβ-(1–42) is more than 10-fold that of Aβ-(1–40). Amyloid fibrils formed in solution are much less toxic.

latter observation suggests that Aβ-(1–42) fibrils are more readily fragmented. The fragmentation greatly facilitates fibril growth because fibrils grow only at their ends [21].

6. Concluding Remarks

Based on the above observations, we propose a novel model for Aβ-membrane interaction as a mechanism for the abnormal aggregation of the protein (Figure 5). Aβ specifically binds to a ganglioside cluster, the formation of which is facilitated by cholesterol. The cluster can be formed also by late endocytic dysfunction [43]. The Aβ undergoes a conformational transition from an α-helix-rich structure to a β-sheet-rich one with increasing protein density on the membrane. The β-sheet form serves as a seed for the formation of amyloid fibrils, which are more toxic than and have different structures from those formed in solution. Depending on ganglioside contents in the membrane, toxic soluble oligomers may also be generated. The amyloidogenic activity of Aβ-(1–42) is more than 10-fold that of Aβ-(1–40), although the initial interaction with gangliosides is similar between the two proteins.

This model can explain roles of various risk factors in the pathogenesis of AD, especially from a viewpoint of lipid metabolism. Both the aging and the apolipoprotein E4 allele are strong risk factors for developing AD [44]. The amount of cholesterol in the exofacial leaflets of the synaptic plasma membrane increases in aged [45] as well as apolipoprotein E4-knock-in [46] mice. GM1 clustering occurs at presynaptic neuritic terminals in mouse brains in an age-dependent manner [47]. Diet-induced hypercholesterolemia accelerates the amyloid pathology in a transgenic mouse model [48]. A link between cholesterol, Aβ, and AD has been reported [49, 50]. Human AD brains also show abnormality in lipid metabolism in accordance with our model [51, 52]. That is, significant increase in GM1 was reported in Aβ-positive nerve terminals from the AD cortex [51], and lipid rafts from the frontal cortex and the temporal cortex of AD brains were also found to contain a higher concentration of GM1 compared to an age-matched control [52]. It should be noted that, in addition to these modulations of Aβ aggregation by lipids, Aβ also in turn regulates lipid metabolism [53].

The 10-fold higher amyloidogenic activity of Aβ-(1–42) is in accordance with the facts (1) that genetic mutations in the presenilins causing early-onset AD increase the level of Aβ-(1–42) [54] and (2) that the protein is the major species in diffuse plaques, the earliest stage in the deposition of Aβ [55].

In conclusion, in addition to other biochemical cascades, a complex purely physicochemical cascade linked to lipid metabolism (Figure 5) appears to be also involved in the process of Aβ aggregation. Inhibition of one of these steps would be a promising strategy for AD therapy.

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